Restricted Cross-Reactivity of Hybrid Capture 2 with Nononcogenic Human Papillomavirus Types[1]

Philip E. Castle,[2] Mark Schiffman, Robert D. Burk, Sholom Wacholder, Allan Hildesheim, Rolando Herrero, M. Concepcion Bratti, Mark E. Sherman, and Attila Lorincz

Division of Cancer Epidemiology and Genetics, National Cancer Institute, Bethesda, Maryland 20892 [P. E. C., M. S., S. W., A. H., M. E. S.]; Albert Einstein College of Medicine, Bronx, New York 10461 [R. D. B.]; Proyecto Epidemiologico Guanacaste, San Jose, Costa Rica [R. H., M. C. B.]; and Digene Corporation, Gaithersburg, Maryland 20878 [A. L.]

Abstract

Hybrid Capture 2 Test using probe B (HC2-B) is a clinical test for the detection of 13 human papillomavirus (HPV) types associated with cervical cancer (oncogenic types), but the potential clinical significance of HC2-B cross-reactivity with untargeted (nononcogenic) HPV types has not been fully evaluated. Thus, HC2-B test results on 954 clinical cervical specimens from a population-based natural history study of HPV in Costa Rica were compared with the data from testing of the same specimens twice by HPV type-specific MY09/MY11 L1 consensus primer PCR. Specimens positive by PCR for single HPV types not targeted by HC2-B were used for determining type-specific cross-reactivity. Effects of cross-reactivity on clinical performance were estimated by calculating sensitivity and specificity with and without cross-reactivity for the detection of high-grade cervical lesions. HC2-B tested positive for single infections by untargeted (cross-reactive) types 11, 53, 61, 66, 67, 70, 71, and 81. Cross-reactivity was strongly associated with PCR signal strength ($P_{Trend} = 0.0001$) and cervical abnormalities ($P = 0.0002$, Pearson χ^2). We estimated that HC2-B cross-reactivity resulted in minor changes in screening performance. Clinical sensitivity increased from 84.3% to 87.9%, clinical specificity decreased from 89.6% to 88.1%, and referral rates increased from 11.7% to 13.2% for detection of ≥cervical intraepithelial neoplasia grade 2. The clinical effect of cross-reactivity varied by cytologic interpretation. Among women with normal cytologic interpretations, cross-reactivity significantly improved the accuracy of identifying cytologically nonevident histology of ≥cervical intraepithelial neoplasia grade 2 because of increased sensitivity with maintained specificity. However, among women with equivocal or mildly abnormal cytologic interpretations, cross-reactivity decreased the accuracy of HPV testing because of substantial decreases in specificity. In summary, cross-reactivity with nononcogenic HPV types had little effect on the overall clinical performance of HC2-B as a general screening test, but reduction of cross-reactivity might improve the performance of HPV testing for triage of equivocal or mildly abnormal cytologic interpretations.

Introduction

HC2[3] test is a clinical test for HPV DNA that uses two different probe sets: probe set A directed against nononcogenic HPV types 6, 11, 42, 43, and 44 (which cause cervical condylomata); and probe set B directed against HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 [which cause virtually all cervical cancer (oncogenic HPV); Refs. 1–3]. HPV DNA detection by HC2-B has been shown to be a sensitive (88.4%) and reasonably specific test (89.0%) for CIN2, CIN3, and cancer (≥CIN2; Ref. 4). HC2-B is also a clinically useful test (96.3% sensitivity) for detecting underlying cervical precancerous and cancerous lesions among women diagnosed with equivocal Pap smears (5).

Previous studies have suggested that HC2-B may cross-react with HPV types either not associated or with undetermined associations with cancer (nononcogenic HPV).[4] One investigation of 208 clinical specimens found that HC2-B cross-reacted with HPV types 53, 66,[4] 67, 71 (AE8, CP8061), 73, and AE6 (CP6108; Ref. 6). A second investigation of 448 clinical specimens found that HC2-B cross-reacted with HPV types 6, 11, 26, 40, 42, 66, 83, and 84 (7).[5] Although these HPV types can cause cytologic abnormalities often detected by repeat Pap screening, these infections rarely if ever progress to cancer. The clinical implication of detecting these "nononcogenic types" by HC2-B has not been evaluated.

Accordingly, we compared HC2-B results to repeat testing by HPV type-specific MY09/MY11 L1 consensus primer PCR on 954 enrollment specimens from a population-based natural history study of HPV and cervical cancer in Costa Rica. Our aims were to confirm the extent of cross-reactivity of HC2-B with nononcogenic HPV types and to gauge the potential clinical significance of this cross-reactivity on the performance of

Received 2/4/02; revised 6/5/02; accepted 7/4/02.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] Supported by a series of National Cancer Institute contracts. In addition, this work was supported in part by NIH Grant CA78527 (to R. D. B.). A. L. is the Chief Scientific Officer of Digene and holds Digene stock and stock options.
[2] To whom requests for reprints should be addressed, Division of Cancer Epidemiology and Genetics, National Cancer Institute, 6120 Executive Boulevard, MSC 7234, Bethesda, MD 20892. Phone: (301) 435-3976; Fax: (301) 402-0916; E-mail: pc95p@nih.gov.

[3] The abbreviations used are: HC2, Hybrid Capture 2 Test; HC2-B, Hybrid Capture 2 Test using probe B; HPV, human papillomavirus; CIN, cervical intraepithelial neoplasia; RLU, relative light unit(s); HSIL, high-grade squamous intraepithelial lesion; CI, confidence interval; ASC, atypical squamous cell.
[4] There are inadequate data about the oncogenicity of some HPV types including HPV 66. However, for the purposes of our study, we decided to use the oncogenic definitions that are commonly assumed in the literature.
[5] L. Ho, personal communication.

HPV testing for general screening and triage of mildly abnormal cytology.

Materials and Methods

Study Population

A National Cancer Institute-sponsored, National Cancer Institute- and local institutional review board-approved population-based cohort study of HPV and cervical neoplasia was established in Guanacaste, Costa Rica, in 1993–1994 (8, 9). At enrollment, 10,049 women of the 11,742 women identified in a door-to-door survey residing in randomly chosen censal segments of Guanacaste agreed to visit one of our study clinics and participated in the enrollment interview. Pelvic examinations were performed on 9,175 women, excluding virgins ($n = 583$) and those women unwilling or unable to undergo an exam ($n = 291$). Twenty-eight of 31 supplemental cervical cancer cases, identified from major centers to which Guanacaste residents are referred for diagnosis and treatment, were alive and agreed to participate. Thus, enrollment cervical specimens were collected from 9,203 women.

Specimen Selection

HC2-B was performed on cervical specimens collected with a dacron swab that was placed in 1.0 ml of specimen transport medium (Digene Corp., Gaithersburg, MD) and stored frozen until tested for HPV DNA (8, 9).

Testing was done on a stratified sample of the entire cohort ($n = 1119$) to evaluate screening performance and determine the optimal threshold for a positive test for HC2-B (4). Strata were defined by cytologic interpretation, the results of a previous Hybrid Capture Tube Test (the less sensitive predecessor to HC2-B), and sexual behavior. Based on these strata, the data were extrapolated from the test sample to the entire study population. We previously reported the results of a population-based study of HPV infection and cervical neoplasia in Costa Rica in which MY09/11 consensus primers (with HMB01; Ref. 10) and AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT) were used for HPV DNA detection ($n = 3063$; 3013 valid tests, *i.e.*, those tests with a positive amplification result using human β-globin primers indicating specimen adequacy for PCR testing; Ref. 9). Subsequently, we have retested the enrollment cervical specimens from the entire cohort using a more sensitive MY09/11 assay that used AmpliTaq Gold DNA polymerase (Perkin-Elmer Cetus; $n = 9203$; 9148 valid tests; Ref. 11). This assay was more sensitive for HPV DNA detection than the earlier MY09/11 assay due either to the switch from AmpliTaq to AmpliTaq Gold or to the 3-fold greater concentration of AmpliTaq Gold compared to AmpliTaq. Of the 1119, a set of 954 specimens had valid tests for HC2 and for both PCR assays; PCR test results were combined such that a positive test for a HPV type by either PCR test was considered positive to maximize analytic sensitivity.

HPV DNA Detection

HC2-B Testing. HPV DNA testing by HC2 used only probe set B, containing RNA probes for 13 cancer-associated (oncogenic) HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68; Ref. 12). Signal strengths in RLU were compared with 1 pg/ml HPV type 16 DNA-positive controls (RLU/PC), and specimens with ≥ 1 RLU/PC were considered HPV DNA positive. HC2-B was performed masked to the clinical or PCR results at Digene Corp. according to the manufacturer's specifications.

PCR Testing. Specimen preparation for PCR is detailed elsewhere (11, 13). Briefly, an aliquot of the specimen transport medium specimen was lysed, DNA was precipitated by ammonium acetate/ethanol solution and then pelleted by centrifugation. The DNA pellet was suspended in 10 mM Tris (pH 7.5) and 0.1 mM EDTA and stored frozen until used.

Amplification by MY09/11 with AmpliTaq has been reported previously (13). The composition of the two PCR reactions was the same, except that 7.5 units of TaqGold were used instead of 2.5 units of AmpliTaq DNA polymerase. Thermocycling conditions for the AmpliTaq and TaqGold reactions have been published previously (11, 13). A 100-cell copy SiHa DNA-positive control, a 2-cell copy SiHa DNA-positive control, and a 100-cell copy HuH7 DNA-negative control were used per every 48 specimens tested.

PCR products were analyzed by gel electrophoresis and then transferred to nylon filters. The filters were hybridized overnight with radiolabeled generic probes for HPV (HPV types 11, 16, 18, 51, 73, and 81 combined) as has been described previously (14). Two observers evaluated the signal strength of the PCR products (14). Dot blot hybridization for HPV type-specific detection was conducted as described in detail elsewhere (13). Briefly, all PCR products were hybridized with type-specific probes for HPV types (11, 13) 2, 6, 11, 13, 16, 18, 26, 31–35, 39, 40, 42–45, 51–59, 61, 62, 64, 66–70, 71 (AE8), 72, 73, 81 (AE7), 82 (W13B), 83 (PAP291), 84 (PAP155), 85 (AE5), 89 (AE6), AE2 (IS39), AE9, and AE10. Probes for HPV types 2, 13, 34, 42–4, 57, 62, 64, 69, 74, 82 (W13B), and AE9 were combined in dot blot hybridizations for detection of rare nononcogenic types (dbmix). A specimen was considered HPV positive but uncharacterized if it tested positive for HPV DNA by the generic probe set but was not positive for any specific probe. Three experienced investigators interpreted each dot blot result, and discrepancies were resolved by consensus.

Statistical Analysis

We defined oncogenic HPV types as those 13 types targeted by HC2-B, the types also most commonly found in cervical cancer tissue collected worldwide by Bosch *et al.* (1). For simplicity and consistency with other literature, we combined all other types into the category of nononcogenic types. We acknowledge that there is incomplete data on the oncogenic properties of some HPV types due to their low prevalence and that some nononcogenic types may very rarely cause cancer. We note that 6 of 108 cases of CIN3 and cancer (including the supplemental cancer cases) in this study had only nononcogenic HPV types (1 case with HPV types 11 and 84, 1 case with HPV type 66, and 4 cases that were uncharacterized) detected, but 5 others among the 108 cases were negative by PCR, suggesting that any or all of these 11 cases might be falsely negative for oncogenic types.

One goal was to assess the cross-reactivity between HC2-B and nononcogenic HPV types. To this end, we excluded from this analysis all specimens positive for oncogenic HPV types by either of the PCR tests. Specimens infected by a single nononcogenic HPV type as detected by PCR were used to ascertain which untargeted types were detected by HC2-B. We used two different measurements to assess the role of viral load in the cross-reactivity of HC2-B with nononcogenic HPV types. First, restricting to single cross-reactive HPV type infections, we tested the association using χ^2 test for trend of HC2-B positivity with PCR signal strength (on a scale of 1–5, with weakest = 1 and strongest = 5), a qualitative index of viral load

that has been correlated with quantitative TaqMan PCR[6] (refer to Table 3). Second, using the same restriction to single cross-reactive HPV type infections, we tested the association of HC2-B positivity with equivocal, mildly abnormal cytologic interpretations, or more severe cytologic interpretation using a Pearson χ^2 test. Cytomorphologic abnormalities interpreted as either equivocal or mildly cervical abnormalities are considered to be the result of productive HPV infection. A previous study demonstrated that HPV viral loads in cervical specimens from women with cytologic abnormalities were higher than in those specimens from infected women in the absence of cytologic abnormalities (4). Thus, we used the presence of cervical abnormalities as another indicator of higher viral loads.

Of the 954 women with all three HPV DNA tests, a subset of 893 women (excluding women with hysterectomies and women who were supplemental cancer cases) whose specimens were originally selected for HC2-B testing based on six sampling strata (4) were used to estimate the performance of HC2-B for the detection of ≥CIN2 (CIN2, CIN3, or cancer). Nine histologically unconfirmed cases of cytologic high-grade lesions were included. A cytologic diagnosis of HSILs overlaps with histological diagnoses of high-grade lesions, CIN2 and CIN3. In clinical practice, a HSIL diagnosis from a Pap smear is referred to colposcopy, where biopsies of visible lesions are taken for histological diagnosis. Cytologic interpretations without histological confirmation are considered unconfirmed diagnoses, but in these few cases, multiple cytologic techniques and review indicated HSIL. The performance for the detection of ≥CIN3 (CIN3 or cancer) was also estimated.

To calculate assay sensitivity and specificity, sampling strata were reconstituted for an estimation of population-wide percentages of HC2-B positivity for nonpregnant, sexually active women without hysterectomies ($n = 8551$). To examine the effects of cross-reactivity on HC2-B performance, specimens positive by PCR for only nononcogenic types and HC2-B positive were treated as if these specimens were HC2-B negative, and sensitivity and specificity calculations were repeated. Clinical performance estimates were stratified on age (<30 years old, ≥30 years old), number of lifetime sexual partners (<5 partners, ≥5 partners), and cytology (normal *versus* equivocal or mildly abnormal). Finally, a measurement of test accuracy, Youden's index, was calculated (Youden's index equals percentage sensitivity + percentage specificity − 100%; a test with perfect sensitivity and specificity has a Youden's index of 100%) with 95% CIs as a summary statistic for the clinical performance with and without HC2-B cross-reactivity (15). Referral rates were based on the assumption that a positive HC2-B test for HPV DNA would be the basis of colposcopic referral. Differences between estimated Youden's indices and referral rates with and without cross-reactivity were tested for statistical significance by calculating Z statistics.

Results

There were 954 specimens tested by HC2, tested by MY09/11 PCR using AmpliTaq, and tested by MY09/11 PCR using AmpliTaq Gold in this stratified random sample of the Guanacaste population skewed toward HPV-infected women. Of the 954 specimens, 719 (75.4%) were positive for any HPV type, and 131 specimens (13.7%) were positive only for nononcogenic HPV types. Table 1 shows the prevalence of oncogenic HPV types and the percentage of HC2-B positivity in this study group. Table 2 shows the type-specific detection by HC2-B among specimens PCR positive only for nononcogenic HPV types. Overall, HC2-B was positive for 39 of 131 (29.8%; 95% CI, 22.1–38.4%) infections by one or more nononcogenic types in the absence of an oncogenic HPV infection. HC2-B cross-reacted with 12 single infections caused by nononcogenic HPV types 11, 53, 61, 66, 67, 70, 71, and 81; 10 of these infections were positive for the same HPV type by both PCR tests, one was HPV positive but untyped by the AmpliTaq reaction and typed as HPV type 61 by AmpliTaq Gold reaction, and one was HPV negative by AmpliTaq and typed as HPV type 71 by AmpliTaq Gold reaction. HC2-B also cross-reacted with infections by dbmix (a composite of nononcogenic types) and infections with uncharacterized HPV types and with a joint infection by types 54 and AE2.

Table 1 Number of specimens positive for oncogenic HPV types (single and multiple infections) and the percentage detected by HC2-B among the 954 subjects selected for these analyses[a]

HPV type	No. of specimens positive by combined PCR	% HC2 positive
16	125	84.0
18	35	74.3
31	38	89.5
33	22	86.4
35	19	84.2
39	38	92.1
45	30	76.7
51	56	78.6
52	57	79.0
56	33	93.9
58	57	82.5
59	14	92.9
68	11	100.0

[a] To maximize the statistical power of this analysis, specimens were selected as part of a stratified sample at high-risk for HPV infection. Strata were defined by cytologic diagnoses, the results of a previous Hybrid Capture Tube Test (the less sensitive predecessor to HC2-B), and sexual behavior.

We examined the association of PCR signal strength and an abnormal diagnosis with HC2-B positivity, restricted to single infections by cross-reactive types ($n = 40$). Positive HC2-B test results ($n = 12$) were associated with greater PCR signal strength ($P_{\text{Trend}} = 0.0001$; Table 3). Similarly, positive HC2-B test results were associated with equivocal or more severe cytology (≥ASC) compared with negative cytology ($P = 0.0002$, Pearson χ^2; Table 4). Among women with ASC, HC2-B cross-reactivity was still associated with PCR signal strength ($P_{\text{Trend}} = 0.0008$), and all 8 ≥ASC specimens with a PCR signal strength of 4 or 5 tested positive by HC2-B.

To gauge the effects of type cross-reactivity on clinical performance, we compared the sensitivity and specificity of HC2-B based on its actual performance with the theoretical performance of HC2-B in the absence of the cross-reactivity with nononcogenic HPV types (Table 5). The performance of HC2-B with and without cross-reactivity was assessed at two thresholds, ≥CIN2 and ≥CIN3. Using a positive HC2-B test for HPV DNA as the basis for colposcopic referral, we estimated a referral rate of 13.2%, with 87.9% sensitivity and 88.1% specificity for ≥CIN2 and 94.1% sensitivity and 87.6% specificity for ≥CIN3 for HPV DNA detection by HC2-B as it currently performs. We estimated a theoretical referral rate of 11.7%, with 84.3% sensitivity and 89.6% specificity for ≥CIN2 and 92.9% sensitivity and 89.2% specificity for ≥CIN3 for HPV DNA detection by HC2-B in the absence of cross-

[6] Dr. P. E. Gravitt, personal communication.

Table 2 Detection of nononcogenic HPV types by HC2-B as determined by combined type-specific PCR

Single infections are bold for emphasis.

HPV type	No. of single type infections (PCR)	HC2-B detection	
		Positive	% detection
6	3	0	0.0
11	2	1	50.0
26	2	0	0.0
32	2	0	0.0
40	1	0	0.0
53	7	2	28.6
54	2	0	0.0
55	2	0	0.0
61	7	1	14.3
66	4	3	75.0
67	1	1	100.0
70	6	2	33.3
71	11	1	9.1
72	2	0	0.0
73	2	0	0.0
81	2	1	50.0
83	2	0	0.0
84	1	0	0.0
85	1	0	0.0
AE2	0	0	
89 (AE6)	1	0	0.0
AE10	0	0	
Mix[a]	7	2	28.6
Uncharact.[b]	19	4	21.1
6, 70	2	2	100.0
6, 81	1	1	100.0
11, 73	1	0	0.0
11, 84	1	1	100.0
26, 73	1	0	0.0
40, 61	1	0	0.0
53, 55	1	0	0.0
53, 70	1	1	100.0
53, AE10	1	1	100.0
53, AE2	2	0	0.0
53, 85	2	1	50.0
53, AE6	1	1	100.0
53, 84	2	1	50.0
54, AE2	1	1	100.0
54, dbmix	1	0	0.0
55, 81	2	0	0.0
55, dbmix	1	0	0.0
61, 66	1	1	100.0
67, 85	1	1	100.0
70, 83	1	0	0.0
AE10, dbmix	1	0	0.0
AE2, dbmix	1	0	0.0
85, AE6	1	0	0.0
11, 53, dbmix	1	1	100.0
53, 70, 73	1	1	100.0
53, AE10, dbmix	1	1	100.0
61, 70, dbmix	1	1	100.0
61, 70, 84	1	1	100.0
61, 71, AE10	1	0	0.0
66, AE10, Mix	1	0	0.0
67, AE6, AE8	1	0	0.0
6, 53, 70, AE6	1	1	100.0
6, 54, 70, 73	1	1	100.0
53, 66, AE2, AE8	1	1	100.0
66, 70, PAP291, Mix	1	0	0.0
53, 61, AE2, AE7, Mix	1	1	100.0
67, AE5, AE6, AE8, Mix	1	0	0.0
61, 67, AE5, AE6, AE8, Mix	1	0	0.0
67, 70, AE5, AE6, AE8, Mix	1	0	0.0
Total	131	39	

[a] HPV types 2, 13, 34, 42–44, 57, 62, 64, 69, 74, W13B (82), and AE9.
[b] HPV positive by the general probe but untyped.

Table 3 A comparison of HC2-B test results and PCR signal strength for single infections by HPV types 11, 53, 61, 66, 67, 70, 71, and 81

Column percentages are provided. $P_{Trend} = 0.0001$.

	PCR signal strength					Total
	1	2	3	4	5	
HC2 negative	6	10	9	3	0	28
	100.0%	90.9%	75.0%	37.5%	0.0%	
HC2 positive	0	1	3	5	3	12
	0.0%	9.1%	25.0%	62.5%	100.0%	
Total	6	11	12	8	3	40

Table 4 A comparison of HC2-B test results for single infections by HPV types 11, 53, 61, 66, 67, 70, 71, and 81 to normal cervical diagnosis *versus* any abnormal cervical diagnosis (ASCs or more severe, ≥ASC)

Column percentages are provided. $P = 0.0002$, Pearson χ^2; n.b., there were no women with hysterectomies included in this analysis.

	Diagnosis		Total
	Normal	≥ASC[a]	
HC2 negative	18	10	28
	100.0%	45.5%	
HC2 positive	0	12	12
	0.0%	54.5%	
Total	18	22	40

[a] Included two low-grade squamous intraepithelial lesions and one HSIL (CIN2) among the HC2 negatives and six LSILs and one supplemental cancer among the HC2 positives.

reactivity with nononcogenic HPV types. Youden's index, which assumes that clinical sensitivity and specificity are equally important attributes of a screening test, was no different for the detection of ≥CIN2 by HC2-B with HC2-B cross-reactivity (Youden index = 75.9%; 95% CI, 70.8–81.1%) compared with detection of ≥CIN2 by HC2-B without HC2-B cross-reactivity (Youden index = 73.9%; 95% CI, 68.3–79.4%). Likewise, there was virtually no difference in Youden's index for the detection of ≥CIN3 by HC2-B with cross-reactivity compared with HC2-B without cross-reactivity.

Cross-reactivity of HC2-B with nononcogenic HPV types had a minor impact on clinical test performance among women under the age of 30 years compared with women 30 years and older. We estimated that HC2-B cross-reactivity would lead to a slightly greater (nonsignificant) increase in referral rates (22.0% *versus* 19.0%) for women under the age of 30 years compared with women 30 years and older.

The effects of cross-reactivity on screening performance appeared to have different impact in strata defined by cytology. Although there were no appreciable differences in referral rates among women with normal cytology with or without cross-reactivity, the Youden's index for ≥CIN2 detection was greater with cross-reactivity (42.5%; 95% CI, 37.3–47.7) than without cross-reactivity (30.3%; 95% CI, 25.1–35.5; $P < 0.0001$) as the result of greater sensitivity. Among women with abnormal cytology (equivocal or mildly abnormal), the referral rates were significantly greater with cross-reactivity (33.9%; 95% CI, 31.2–36.6%) than without cross-reactivity (26.1%; 95% CI, 23.6–28.6%; $P < 0.0001$), and Youden's index for ≥CIN2 detection was significantly lower with cross-reactivity (59.1%; 95% CI, 53.9–64.3%) than without cross-reactivity (67.0%; 95% CI, 61.8–72.1%; $P = 0.02$) as the result of reduced

Table 5 Sensitivity and specificity of HC2-B for high-grade CIN with or without cross-reactivity for nononcogenic types

A ≥CIN2 diagnosis includes any diagnosis of CIN2, CIN3, or cancer. A ≥CIN3 diagnosis includes CIN3 or cancer. For this table, abnormal cytology refers to women with a Pap test interpreted as either equivocal or mildly abnormal. Youden's indices (YI)[a] with 95% CIs are presented as summary statistics of clinical performance. Referral is the percentage referred to treatment based on a positive HC2-B test. Bold numbers indicate statistical significance.

Disease end point	Subgroup	*N* (cases)[b]	With cross-reactivity				Without cross-reactivity			
			Sensitivity (%)	Specificity (%)	YI (%) (95% CI)	Referral (%)	Sensitivity (%)	Specificity (%)	YI (%) (95% CI)	Referral (%)
≥CIN2	All	8551 (140)	87.9	88.1	75.9 (70.6–81.3)	13.2	84.3	89.6	73.9 (68.1–79.6)	11.7
	<30 yrs of age	2318 (36)	91.7	79.1	70.7 (65.4–76.1)	22.0	91.7	82.1	73.8 (68.4–79.2)	19.0
	≥30 yrs of age	6233 (104)	86.5	91.0	77.6 (72.2–82.9)	10.3	81.7	91.9	73.7 (68.3–79.0)	9.3
	<5 sex partners	7999 (123)	88.6	88.6	77.3 (71.9–82.6)	12.6	85.4	90.1	75.5 (70.1–80.9)	11.0
	≥5 sex partners	547 (17)	82.4	87.6	69.9 (64.5–75.3)	14.6	76.5	88.7	65.2 (59.8–70.5)	13.4
	Normal cytology	7127 (8)	50.0	92.5	**42.5 (37.1–47.8)**	7.6	37.5	92.8	**30.3 (25.0–35.7)**	7.2
	Abnormal cytology	1192 (13)	92.3	66.8	**59.1 (53.7–64.4)**	33.9	92.3	74.6	**67.0 (61.6–72.3)**	26.1
≥CIN3	All	8551 (85)	94.1	87.6	81.8 (77.2–86.4)	13.2	92.9	89.2	82.1 (77.1–87.1)	11.7
	<30 yrs of age	2318 (21)	95.2	78.6	73.9 (69.3–78.5)	22.0	95.2	81.7	76.9 (72.3–81.5)	19.0
	≥30 yrs of age	6233 (64)	93.8	90.6	84.4 (79.8–89.0)	10.3	92.2	91.6	83.7 (79.1–88.3)	9.3
	<5 sex partners	7999 (77)	93.5	88.2	81.7 (77.1–86.3)	12.6	92.2	89.7	82.0 (77.3–86.5)	11.0
	≥5 sex partners	547 (8)	100.0	86.6	86.6 (82.0–91.2)	14.6	100.0	87.9	87.9 (83.3–92.5)	13.4
	Normal cytology	7127 (3)	66.7	92.5	**59.1 (54.5–63.7)**	7.6	33.3	92.8	**26.1 (21.5–30.7)**	7.2
	Abnormal cytology	1192 (8)	100.0	66.6	**66.6 (62.0–71.2)**	33.9	100.0	74.4	**74.4 (69.8–79.0)**	26.1

[a] Youden's index = % sensitivity + % specificity − 100%.
[b] Reconstituted population numbers based on a stratified sample of 893 women.

specificity. These conclusions did not differ when a disease end point of ≥CIN3 was used.

Discussion

In our study, we defined HC2-B cross-reactivity with nononcogenic HPV types by testing specimens twice by MY09/11 PCR and accepting a positive test by either as positive. The primary advantages of combining PCR testing in this manner were the sensitive detection of oncogenic type infections and increased certainty for the detection of nononcogenic HPV types. Using this approach, we found HC2-B to be cross-reactive with HPV types 11, 53, 61, 66, 67, 70, 71, and 81. Of note, only a single infection by HPV type 61 and another by HPV type 71 as identified by the AmpliTaq Gold reaction were not confirmed by AmpliTaq reaction. Thus, we confirmed in a population-based study the cross-reactivity of HC2-B with HPV types 53, 66, 67, and 71 as found by Peyton *et al.* (6) and additionally with HPV type 11 as found by Terry *et al.* (7). We found cross-reactivity with dbmix, a combination of less prevalent non-oncogenic types, which includes type 42 as found by Terry *et al.* (7). HC2-B also cross-reacted with specimens infected with uncharacterized types, and one multiple infection of HPV types 54 and AE2 was also HC2-B positive. We did not find cross-reactivity with single-type infections of HPV type 73 and AE6 (6), nor did we find cross-reactivity with HPV types 6, 26, 40, 83, and 84 (7).

Population screening characteristics for the detection of ≥CIN2 in the analytic subset of 893 women (87.9% sensitivity and 88.1% specificity) were similar to the previously published sensitivity/specificity characteristics for the detection of ≥CIN2 in the entire stratified subsample of 1119 women (88.4% sensitivity and 89.0% specificity; Ref. 4). Excluding those HC2-B-positive results due to nononcogenic HPV type infections alone (11.3% of all HC2 results and approximately 1.3% of the total population) resulted in a slight decrease in referral rates with a concomitant slight decrease in sensitivity and slight increase in specificity for the detection of high-grade cervical lesions.

The influence of cross-reactivity on clinical performance was (nonsignificantly) greater among women under the age of 30 years than for women 30 years and older, as indicated by the estimated increase in referral rates of 3.0% in the younger women compared with a 1.0% increase in the older women. Increased referral rates were entirely the consequence of a decrease in clinical specificity. This is perhaps not surprising, given the greater prevalence of HPV infections among younger women. Surprisingly, we did not find a greater increase in referral rates as the result of HC2-B cross-reactivity among women with ≥5 lifetime sexual partners compared with the overall population.

The effects of cross-reactivity varied by cytologic interpretation. Interestingly, cross-reactivity improved the screening performance of HC2-B among cytologically normal women as the result of increased sensitivity for histological ≥CIN 2, without substantial loss of specificity. At the low viral loads found among cytologically normal women, the advantages of clinical sensitivity predominated. This could be relevant when HC2-B testing is being considered as an adjunctive primary screening test to increase the accuracy of cytology.

However, among women with equivocal or mildly abnormal cytologic interpretations, cross-reactivity decreased the accuracy of HPV testing because of substantial decreases in specificity without sufficient compensatory gains in sensitivity. In analyses restricted to HPV-positive women, average viral loads correlate with the probability of a cytologic abnormality. Higher viral loads of nononcogenic HPV types increase cross-reactivity such that the specificity of testing decreases. As a corollary, our results indicate that cross-reactivity tends to decrease the utility of HPV testing in the triage of equivocal and slightly abnormal cytology (Ref. 16).

The theoretical decrease in clinical sensitivity for ≥CIN2, the standard clinical threshold for treatment in the United States and Costa Rica, was greater (3.6%) when cross-reactivity was subtracted than it was for the more severe ≥CIN3 (1.2%) diagnosis. Perhaps this result should be expected because CIN2 could be caused by other uncharacterized HPV types cross-reactive to HC2, and CIN2 is more likely to regress to nor-

malcy. In contrast, CIN3 is a precancer diagnosis and thus is less likely to be caused by a nononcogenic HPV.

We offer two possible explanations for HC2-B cross-reactivity with nononcogenic types. First, HPV types 53, 66, 67, and 70 (but not HPV types 11, 61, 71, and 81) belong to three HPV phylogenetic clades that contain most of the oncogenic types. Sequence conservation between some oncogenic types and these genetically related cross-reactive types may account for lack of perfect fidelity of the HC2-B probe set, which may explain the predilection for HC2-B to cross-react with these types. Second, cross-reactivity of HC2-B with any nononcogenic type or uncharacterized type could be attributable to misclassification of oncogenic HPV DNA status by PCR. However, by using the results of duplicate MY09/11 PCR tests, we attempted to minimize this possibility by maximizing our sensitivity for the detection of all HPV types. Only 16 of the 450 PCR-negative specimens (4%) were positive by HC2-B, compared with 4 of 22 single infections (18%) by HPV types 11, 61, 71, and 81 detected by HC2-B, which suggests that PCR missed very few oncogenic infections and that misclassification alone cannot explain these findings. By the same reasoning, chance is also unlikely to explain HC2-B cross-reactivity with the more common nononcogenic types, particularly HPV type 53. HC2-B cross-reactivity among the uncharacterized types may also be attributable to one of the many HPV types not included in the standard probe set.

Specimens with higher viral loads of these cross-reactive, nononcogenic types, as measured by multiplicity of infection [n.b., HC2-B detected 12 of 61 single-type nononcogenic infections (19.7%; 95% CI, 10.6–31.8%) *versus* 21 of 44 multiple-type nononcogenic infections (47.7%; 95% CI, 32.5–63.3%)], PCR signal strength, and cytologic abnormalities, were more likely to result in false positive detection by HC2-B. Aside from chance, differences in type-specific cross-reactivity by HC2-B between studies may be related in part to differences in viral loads of the individual specimens infected with the nononcogenic HPV types.

In summary, we have demonstrated the consequences of HC2-B cross-reactivity with nononcogenic HPV types on its clinical test performance for the detection of high-grade cervical neoplasia. The magnitude of this effect in other populations will be dependent on the relative prevalence of these HPV types compared with the 13 oncogenic types and thus will be region specific. The impact of cross-reactivity will vary with the intended use of the test and the setting. In resource-rich countries such as the United States, the emphasis is often on the detection of all disease. Thus, the trade-off of lesser specificity for greater sensitivity may be a lesser concern. By contrast, in resource-poor settings, greater specificity may be more important because it will result in fewer referrals for treatment but at the cost of some false negatives. The next generation of the Hybrid Capture, Hybrid Capture 3 Test, has been designed for increased assay sensitivity and greater HPV type analytic specificity through the use of type-specific DNA oligonucleotides (11), but its clinical performance has yet to be tested.

Acknowledgments

We gratefully acknowledge the Information Management Services, Inc. group (Rockville, MD) of Julie Buckland, Sabrina Chen, John Schussler, and especially Pei Chao for data management and analytic support. We thank Deidra Kelly (Johns Hopkins University) and Dr. Laurie Mango (Neuromedical Systems, Suffern, New York) for their collaboration in the interpretation of cytological specimens. Reagents and services were supplied or discounted by Cytyc Inc. (Boxborough, MA), National Testing Laboratories (Fenton, MO), Utah Medical (Midvale, UT), and Neuromedical Systems. We offer special recognition for the excellent work of the study staff in Costa Rica, in particular Fernando Cardenas, Manuel Barrantes, Elmer Perez, Lidia Ana Morera, Iris Ugarte, Dr. Mario Alfaro (cytology reading), and Dr. Jorge Morales (colposcopy). We also acknowledge the collaboration of health authorities in Costa Rica for their enthusiastic support of this project and the outreach workers of the Ministry of Health of Costa Rica who carried out the population census for their dedication to the health of the people of Guanacaste.

References

1. Bosch, F. X., Manos, M. M., Munoz, N., Sherman, M., Jansen, A. M., Peto, J., Schiffman, M. H., Moreno, V., Kurman, R., and Shah, K. V. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. International Biological Study on Cervical Cancer (IBSCC) Study Group. J. Natl. Cancer Inst. (Bethesda), *87:* 796–802, 1995.

2. Walboomers, J. M., Jacobs, M. V., Manos, M. M., Bosch, F. X., Kummer, J. A., Shah, K. V., Snijders, P. J., Peto, J., Meijer, C. J., and Munoz, N. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J. Pathol., *189:* 12–19, 1999.

3. Munoz, N. Human papillomavirus and cancer: the epidemiological evidence. J. Clin. Virol., *19:* 1–5, 2000.

4. Schiffman, M., Herrero, R., Hildesheim, A., Sherman, M. E., Bratti, M., Wacholder, S., Alfaro, M., Hutchinson, M., Morales, J., Greenberg, M. D., and Lorincz, A. T. HPV DNA testing in cervical cancer screening: results from women in a high-risk province of Costa Rica. J. Am. Med. Assoc., *283:* 87–93, 2000.

5. Solomon, D., Schiffman, M., and Tarone, R., ALTS Study Group. Comparison of three management strategies for patients with atypical squamous cells of undetermined significance: baseline results from a randomized trial. J. Natl. Cancer Inst. (Bethesda), *93:* 293–299, 2001.

6. Peyton, C. L., Schiffman, M., Lorincz, A. T., Hunt, W. C., Mielzynska, I., Bratti, C., Eaton, S., Hildesheim, A., Morera, L. A., Rodriguez, A. C., Herrero, R., Sherman, M. E., and Wheeler, C. M. Comparison of PCR- and hybrid capture-based human papillomavirus detection systems using multiple cervical specimen collection strategies. J. Clin. Microbiol., *36:* 3248–3254, 1998.

7. Terry, G., Ho, L., Londesborough, P., Cuzick, J., Mielzynska-Lohnas, I., and Lorincz, A. Detection of high risk HPV types by the hybrid capture 2 test. J. Med. Virol., *65:* 155–162, 2001.

8. Herrero, R., Schiffman, M. H., Bratti, C., Hildesheim, A., Balmaceda, I., Sherman, M. E., Greenberg, M., Cardenas, F., Gomez, V., Helgesen, K., Morales, J., Hutchinson, M., Mango, L., Alfaro, M., Potischman, N. W., Wacholder, S., Swanson, C., and Brinton, L. A. Design and methods of a population-based natural history study of cervical neoplasia in a rural province of Costa Rica: The Guanacaste Project. Pan Am. J. Public Health, *1:* 362–375, 1997.

9. Herrero, R., Hildesheim, A., Bratti, C., Sherman, M. E., Hutchinson, M., Morales, J., Balmaceda, I., Greenberg, M. D., Alfaro, M., Burk, R. D., Wacholder, S., Plummer, M., and Schiffman, M. Population-based study of human papillomavirus infection and cervical neoplasia in rural Costa Rica. J. Natl. Cancer Inst. (Bethesda), *92:* 464–474, 2000.

10. Hildesheim, A., Schiffman, M. H., Gravitt, P. E., Glass, A. G., Greer, C. E., Zhang, T., Scott, D. R., Rush, B. B., Lawler, P., Sherman, M. E., Kurman, R. J., and Manos, M. M. Persistence of type-specific human papillomavirus infection among cytologically normal women. J. Infect. Dis., *169:* 235–240, 1994.

11. Castle, P. E., Schiffman, M., Gravitt, P. E., Kendall, H., Fishman, S., Hildesheim, A., Herrero, R., Bratti, M. C., Sherman, M. E., Lorincz, A. T., Schussler, J. E., and Burk, R. D. Evaluation of HPV DNA testing by PCR. J. Med. Virol., *68:* 417–423, 2002.

12. Lorincz, A. T., and Anthony, J. Advances in HPV detection by Hybrid Capture. Papillomavirus Rep., *12:* 145–154, 2001.

13. Qu, W., Jiang, G., Cruz, Y., Chang, C. J., Ho, G. Y., Klein, R. S., and Burk, R. D. PCR detection of human papillomavirus: comparison between MY09/MY11 and GP5+/GP6+ primer systems. J. Clin. Microbiol., *35:* 1304–1310, 1997.

14. Burk, R. D., Ho, G. Y., Beardsley, L., Lempa, M., Peters, M., and Bierman, R. Sexual behavior and partner characteristics are the predominant risk factors for genital human papillomavirus infection in young women. J. Infect. Dis., *174:* 679–689, 1996.

15. Skendzel, L. P., and Youden, W. J. Systematic *versus* random error in laboratory surveys. Am. J. Clin. Pathol., *54:* 448–450, 1970.

16. Wright, T. C., Jr., Cox, J. T., Massad, L. S., Twiggs, L. B., and Wilkinson, E. J., for the 2001 ASCCP-Sponsored Consensus Conference. 2001 Consensus guidelines for the management of women with cervical cytological abnormalities. J. Am. Med. Assoc., *287:* 2120–2129, 2001.